Title
Novel Localized Drug Delivery Methods to Enhance Post Orthodontic Retention

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Publication Date
2020

Peer reviewed|Thesis/dissertation

Novel Localized Drug Delivery Methods to Enhance Post Orthodontic Retention

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THESIS
Submitted in partial satisfaction of the requirements for degree of
MASTER OF SCIENCE

in

Oral and Craniofacial Sciences

in the

GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Novel Localized Drug Delivery Methods to Enhance Post Orthodontic Retention

Siyouneh Novshadian

Abstract

Post-orthodontic relapse remains a challenging and undesirable consequence of orthodontic treatment. Relapse occurs in part due to the presence of relatively immature bone in the direction of tooth relapse that can be readily resorbed by osteoclasts. Because current approaches to retain tooth alignment have well-known caveats, recent studies, including several from our group, have explored the utility of biologic agents such as osteoprotegerin (OPG) in mitigating post-orthodontic relapse. In proof-of-concept studies the repeated submucosal injection of naked OPG resulted in a 3.5-fold increase in post-orthodontic tooth stability. In contrast, the administration of a single dose of OPG-loaded PLGA microbeads contributed only to modest increases in tooth stability. This was attributed to an initial bolus release of OPG from the delivery system. Therefore, the goal of the current study was to design and test *in vitro* other methods of drug delivery for slow sustained release of OPG at optimal concentrations to have clinical efficacy. We tested the hypothesis that optimally constituted OPG-loaded polythelyne glycol (PEG) nanoparticles would result in a slow, sustained, and optimal release of OPG *in vitro*. We also designed and tested a novel method of intraoral drug delivery using thin film devices. The microspheres were loaded with 330**µg of OPG** into PEG microspheres and were placed in PBS and the eluated assayed every day for 28 days. The findings showed gradually decreasing but sustained release of OPG over a 28 day period. Further enhancements in PEG microsphere fabrication and OPG concentrations will be instituted for ongoing studies to optimize the release kinetics. The thin films were constructed from polyethylene glycol, customized to fit onto the rat palate

and loaded with 90μg of OPG. Following further enhancements to the PEG microspheres, thin film fabrication, and optimization of OPG release kinetics, these delivery systems will be tested for their efficacy in enhancing tooth stability in a rat model for orthodontic relapse.

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Introduction

The stability of teeth following orthodontic treatment remains a challenge due to the natural tendency for teeth to move towards their original positions. Orthodontic relapse is an undesirable tendency for teeth to return to or move towards their pretreatment positions after having been moved through bone. This instability is a significant limitation to treatment outcomes due to the negative effects on tooth alignment and esthetics, the high rate at which it occurs, and its impact on patient satisfaction and significant financial burden on patients who require re-treatment. As current methods of enhancing stability, such as retainer wear, rely on long term patient compliance, orthodontic relapse remains a prevalent issue. While the cause(s) of orthodontic relapse remain poorly understood, of the many theorized contributions to relapse, bone quality and maturity is thought to be a major component.[1] Gaining a better understanding of the biologic processes that occur within the bone following orthodontic treatment and during the stabilization period could provide us with direction toward more effective preventive or therapeutic strategies.

According to the widely accepted “pressure-tension” theory of orthodontic tooth movement, the periodontal ligament (PDL) is compressed on the pressure side which is the side in the direction the force is being applied, and thus, the direction of the tooth movement. This compression of the PDL leads to metabolic changes such as the decrease in blood flow and oxygen molecules, and increase in carbon dioxide. These metabolic changes signal specific cytokines to induce osteoclastic activity to begin resorbing the bone in the direction of the force application – a process known as osteoclast mediated resorption.[2,3]

Specifically, Interleukin-1, beta (IL-1β), PGE2, and IL-6 are the cytokines thatplay critical roles in the early processes of activating osteoclast precursor cells via nuclear factor kappa B (RANK) and nuclear factor kappa B ligand (RANKL) proteins.[4] In addition, osteoblastic cells also play a role in promoting more osteoclastic differentiation by synthesizing RANKL.[5] The surface expression of RANKL on osteoblast cells communicates with osteoclastic precursor cells expressing the RANK receptor to induce osteoclast differentiation and activation.[6]

To add to this knowledge of orthodontic tooth movement, a newer theory called the “stretched fiber hypothesis” suggests that the elastic fibers in the PDL should be considered when conceptualizing tooth movement. Given that PDL fibers are of elastic nature, when they are compressed, they exert less force on the adjacent bone. That lack of compression on the adjacent bone may be the cause of bone resorption. This concept aligns with the mechanostat theory in which it is hypothesized that low strain of bone leads to bone resorption.[7]

Simultaneously, osteoblast-mediated bone deposition occurs on the opposite side of the tooth roots in the area from which the tooth moved by similar communications and processes via cytokines. This cascade of events results in tooth movement.[8] Conceptually, in order for relapse to occur towards the pre-treatment tooth position, the newly formed and relatively immature bone deposited by osteoblasts adjacent to the tooth must be resorbed by osteoclasts. Thus, biologics or drugs that contribute to enhanced or accelerated bone synthesis and maturation or drugs that minimize bone resorption by osteoclasts could be of high therapeutic value in increasing post-orthodontic stability of teeth.

Over the years, several biologics that either primarily increase bone synthesis or decrease bone resorption have been used to test the inhibition of tooth movement for increased anchorage or decreased relapse in animal models. These include bisphosphonates,[9] nitric oxide synthase inhibitor,[10] echistatin,[11] MMP inhibitor,[11,12] osteoprotegerin (OPG) gene[13] or OPG protein.[14–17] Of these, several such as bisphosphonates and estradiol have substantial caveats, including potential systemic effects whereas others were not found to have substantial efficacy in modulating tooth movement. In contrast, the use of OPG has been widely studied and has been shown to greatly limit relapse with no discernible systemic effects on long bones.Osteoclast activation is initiated by the binding of RANKL to the RANK receptor found on osteoclast precursor cells.[3] OPG is a known decoy receptor for RANKL and acts as a competitive inhibitor of RANK thereby inhibiting osteoclastogenesis and bone resorption.[3] It was first used to slow down bone resorption by inhibiting RANKL expression of osteoclasts in a study on postmenopausal women.[18] In additional studies, it was shown to reduce bone resorption systemically in several diseases including Paget's disease, rheumatoid arthritis, hypercalcemia of malignancy, osteolytic metastases, postmenopausal bone loss and periodontal disease.[14,18–22] Our previous studies have combined this knowledge with the concept of bone resorption occurring in the direction of tooth movement to evaluate the mechano-modulation of bone modeling using OPG. Specifically, by exploring its potential to alter tooth movement, reduce orthodontic relapse, and enhance anchorage.[14–16]

Once it was noted that OPG could inhibit bone resorption via a systemic approach, its local effects on orthodontic tooth movement were explored in an animal

model utilizing a low dose of 0.5 mg/kg OPG and high dose of 5 mg/kg OPG to test molar anchorage in rats and compared them to control animals receiving Phosphate Buffered Saline (PBS) (Dunn et al., 2007). The animals received orthodontic force application in the form of nickel titanium springs ligated to the maxillary first molars and incisors (Fig. 1) for 28 days with twice weekly injections of either the low or high dose of OPG via local infiltration to the mucosa surrounding the molars. Tooth movement measurements were taken using stone models that were scanned and measured digitally. Measurements were taken pre-treatment, during treatment, and post-treatment. Administration of low and high dose OPG both resulted in a decrease in mesial movement of the first molar when compared to control animals. When compared to controls, the high dose OPG treatment resulted in a 79% decrease in mesial movement of the first molar, suggesting an enhancement in molar anchorage.[14] This inhibition of tooth movement likely resulted from an inhibition of osteoclastogenesis and was supported by the histologic microCT findings showing enhanced bone quality, as well as immunohistochemical analysis and serum TRAP-5b staining in animals treated with OPG vs the controls.

Following this discovery, this group of investigators led by Dr. Sunil Kapila became interested in the ability of OPG to enhance post-orthodontic stability. The same animal model of spring-force application was used, however, OPG was administered following 28 days of tooth movement when the appliances were removed, thus testing post-orthodontic stability and inhibition of relapse. The animals were injected with either 5 mg/kg OPG, 1 mg/kg OPG, or PBS at several time points after appliances were removed, and monitored for 24 days (Fig. 2). In addition to histology, serum Trap-5b

analysis, and microCT, tooth movement measurements were taken every 4 days. Both the high dose and low dose groups showed significant reduction in tooth movement with no significant differences between the high and low dose groups. While the control group experienced 63% relapse, the low and high dose OPG treated groups experienced 24% and 31% relapse, respectively. The histology performed on these animals showed that all three groups experienced a widening of the PDL space at compression sites, which the investigators concluded accounted for the major relapse of 43% seen within the first two days. No osteoclastic activity was observed in this two-day period, which signified that this rebound period is not attributed to bone remodeling, and therefore cannot be altered by bone modulating agents. Once this rebound percentage was accounted for, the adjusted relapse percentage was 3.5 times lower in the low dose and 40-fold lower in the high dose group than in PBS controls (13 and -1.6%). In addition, improvements in bone quality were observed by microCT and histology. The study concluded that OPG enhanced post-orthodontic stability in rats when repeatedly administering either at a high dose of 5 mg/kg or low dose of 1 mg/kg OPG.[15]

A follow-up study was performed to determine the appropriate single submucosal OPG dose when delivered following tooth movement that would inhibit post-treatment relapse while limiting its effects to the site of injection and concomitantly showing no or little systemic effects (Schneider et al., 2015). The same animal model described previously was used, utilizing a single high dose of 1 mg/kg and low dose of 0.1 mg/kg injection after 28 days of tooth movement. This study investigated the local vs distant effects of OPG by comparing molar relapse to incisor relapse given that the

injection was delivered to the mucosa of the molar. The incisors were found to relapse up to 95% after 28 days. In contrast the single injection of 0.1 mg/kg or 1 mg/kg OPG resulted in a dose-dependent reduction in post-orthodontic relapse by 40% and 60%, respectively, at the end of the experimental period, while having little to no effect on the relapse of the incisors.[16] Histology also confirmed an increase in osteoclast presence using Trap5b staining when compared to pre-treatment animals, and a return to baseline presentation for all groups after 24 days suggesting the OPG injected was non-active after 24 days of relapse. MicroCT analysis of the rat femurs at the end of the experimental period showed no significant differences in any of the bone quality measures between the three groups, suggesting OPG does not produce long-lasting systemic effects. The results of this study demonstrate that a single dose of OPG is adequate in significantly mitigating orthodontic relapse with limited effects on tooth movement in the neighboring sites.

Towards a clinical translation of these findings, more controlled, localized, and sustained drug delivery and preferably non-invasive methods are needed to optimize the effects of OPG while maintaining minimal to no systemic effects. Also, although these proof of concept studies cited above found improvements in stability of tooth movement using OPG when compared to controls, they did not generate the desired complete inhibition of relapse. The application of drug delivery methods for modulating orthodontic tooth movement has been explored only very recently.[17]

Microspheres are an emerging advancement of drug delivery methods that have gained attention in the biomedical field due to their biocompatibility and their potential as therapeutic agent carriers.[23] Using these methods, the desired drug release kinetics

can be controlled by parameters such as molecular weight, ratio of polymers, and drug concentration.[24] This approach has been used in one recent study where the rate of release of OPG was regulated over a longer duration of time using OPG embedded in polylactic-co-glycolic acid (PLGA) microspheres. The *in vivo* finding using our model of orthodontic anchorage (Dunn et al., 2007) revealed that that injection of 1 mg/kg of OPG encapsulated in PLGA polymer microspheres enhances molar stability relative to the same dose of unencapsulated OPG, suggesting that the slower release of OPG may be more efficacious than an immediate-release OPG in preserving tooth positional stability. Despite these promising findings, the release of OPG from the microspheres lacked consistency and was relatively quick with 50% OPG released within 2.5 days. Concomitantly, the efficacy of this delivery method was relatively modest with 26% less molar movement relative to that with unencapsulated OPG or vehicle controls. This suggests that alternative drug delivery systems that offer sustained and more constant release profiles of the agent may contribute to increased efficacy in reducing relapse. Additionally, the delivery of the drug via non-invasive methods rather than by injection will likely make the proposed approaches to therapy more acceptable to the patient and clinician over that offered by invasive methods.

The goal of this Master's thesis work was to expand upon previous findings towards clinical applications by fabricating and *in vitro* testing of drug delivering devices in order to achieve a highly regulated slow and sustained release of OPG, and subsequently compare its *in vivo* effects to that immediate-release of OPG. The longer-term goal of our studies is to develop drug delivery systems and subject these to *in vivo*

and clinical trials for use in orthodontic applications in mitigating relapse and modulating tooth movement.

In comparison to PLGA microspheres, polyethylene glycol (PEG) based microparticles offer better control and release dynamics for sustained release of drugs, as has been shown previously.[25–28] Although PLGA microspheres are the most commonly used polymers, they have certain limitations due to the hydrophobic nature of PLGA polymers including protein denaturation and adsorption to their hydrophobicity. For long term release, a hydrophilic bioinert copolymer such as PEG is advantageous given the slower degradation process. Its degradation process is slower due to the inorganic copolymers it contains which make it more difficult for the body to breakdown and thus, release the drug into the system. PEG based microparticles are ideal because of their injectable form, biocompatibility, and the relative simplicity in drug, protein, and cell encapsulation.[25–28]

Briefly, a microfluidic system with rounded fluid channels is used to create monodisperse PEG microparticles that ultimately form microspheres.[29,30] Proteins encapsulated in PEG microspheres exhibit drug release kinetics over a period of time that is ideal for our application, ranging from hours to weeks,[25–27,31] contingent on PEG precursor solution composition. Since this method has already been shown to satisfy our criteria for administration and potentially for optimal release kinetics of anti-relapse therapeutics, we have selected this approach as our primary method to test for fabrication and in vitro testing of drug delivery.

Additionally, the delivery of the drug via non-invasive methods rather than by injection will likely make the proposed approaches to therapy more acceptable to the

patient and clinician over that offered by invasive methods. In this context, the use of nanoengineered membranes is a promising approach to provide sustained release of proteins and small molecules. In addition to their ability to provide sustained release, they can be delivered without implantation and therefore, provide a non-invasive approach. Given the porous mucous membranes of the oral cavity, we anticipate the drug to be released rather easily without implantation. Previous work with these devices have demonstrated release over several months with a highly controlled zero-order release rate (i.e. constant rate drug release).[32,33,34,35,36] Zero-order kinetics enables tight control of target drug concentrations. As opposed to our observations when delivering OPG in PLGA polymer microspheres, this method eliminates the initial bolus excess and the late sub-therapeutic decline, while maximizing the available drug payload.

Briefly, thin film membranes are fabricated by spin- or draw-casting a polymer solution, which generates highly controlled films from 10-100 μm thick. These techniques can be further combined with pore-forming agents or sacrificial templates to generate a wide range of micro- and nano-structures. Such polymeric thin films can then be fabricated into devices with a variety of form factors, which can be suited for delivery in confined spaces (Fig. 4). Thin devices fabricated from biodegradable polymer, polycaprolactone (PCL), can naturally degrade and avoid later invasive device removal. Previous work with these thin films in ocular devices and cell-based therapies have demonstrated good biocompatibility across applications.[37,38,39] In addition, these devices were developed to decouple the material fabrication from the drug formulation, allowing optimization of drug stability and payload in parallel with required release rate (Fig. 5). Our goal is to use these approaches to achieve sustained and constant delivery

of biologics for preserving post-orthodontic tooth stability in our rat model. However, first novel methods of design and delivery need to be established given that thin films have never been tested in the oral cavity. We will first design a device to be placed in an animal tooth movement model in the oral cavity, away from occlusal forces and fastened to hard or soft tissues to prevent dislodging. In this study, we plan to take advantage of the emerging advances in nanoparticle delivery and improve on the previous protocols by testing two new novel methods of delivery: PEG microspheres and thin film devices.

Central Hypothesis

We hypothesize that PEG microspheres and thin film devices are effective methods for localized, non-invasive, and controlled release of agents into oral tissue for use in mitigating orthodontic relapse.

Specific Aims

1. Design and fabricate PEG microsphere delivery system that will result in desirable long-term release kinetics of optimal OPG concentrations over 14 to 30 days for subsequent use in vivo studies using the orthodontic relapse rat model.
2. Undertake initial studies on designing and fabricating thin film devices for intraoral applications in the rat for subsequent in vitro and in vivo testing to deliver optimal sustained release of OPG.

These studies will lay the foundations for future investigations to test the efficacy of these OPG delivery systems in mitigating orthodontic relapse in animal studies and possibly in human trials.

Material and Methods

Materials

Poly(ethylene glycol) dimethacrylate (PEG-DMA) (MW 750), 1- vinyl-2-pyrrolidinone, containing sodium hydroxide as inhibitor (MW 111.14) and 2,2- Dimethoxy -2-phenylacetophenone (MW 256.30) were purchased from Sigma Aldrich (St. Louis, MO). Osteoprotegrin (OPG) (60kDa) was generously provided by Amgen Corporation at a concentration of 18.85 mg/mL with a 100% buffer solvent.

Equipment

A micro BCA assay kit from Thermofisher Scientific was used to quantify the concentration of protein release daily in the supernatant. Assays were performed using a SpectraMaxM2E plate reader.

Microsphere fabrication

OPG in the concentration of 18.85 mg/mL was aliquoted followed by dilution with PBS into three concentrations, 1/10 (1885 µg/mL), 1/50 (377µg/mL), and 1/100 (188.5µg/mL) in order to test for the best concentration of OPG for our purposes. The total amount of OPG loaded into the microspheres was determined by the preferred target release of up to 1mg/kg/day. Because the ideal release is 28 days and an average adult male Sprague-dawley rat weighs 0.350 kg, the total loaded amount of OPG was targeted to be 980 micro-gm. For the first release assay, a 50/50 mixture of a PEG with molecular weight of 750 Da and PBS was used for the fabrication of hydrogels. For the second release assay, a 50/50 combination of high molecular weight

PEGDMA 750 Da and low molecular weight PEGDMA 200 Da diluted to a concentration of 1.5% with PBS was tested. The photoinitiator was made by measuring 100 mg of DMPA on a mg scale for accuracy and dissolved in 1 mL of 1-vinyl-2-pyrolidinone in a vial to make 100 mg/ml. A 50/50 solution of PEG-DMA/OPG was made by adding 500 µliters of 750 mg PEG to 500 microliters of OPG. Then, 100 µliters of photoinitiator was added to the PEG OPG mixture followed by sonication for 5 minutes. A similar process was performed for the second release assay.

Once the hydrogels were fabricated, 5 microliters were retrieved for cell counting. The spheres were then divided equally into 8 separate tubes with 1 million spheres/ml to carry out the assay. Each tube was centrifuged for 5 minutes at 2000 RPM. The solutions were washed twice with PBS. Following, the microspheres were immersed in 275 microliters of PBS, sealed and placed in an incubator shaker set at 37°C for daily sampling of the supernatant to quantify the protein release.

To establish *in vitro* OPG release kinetics, the supernatant was collected daily for 28 days. The tubes were centrifuged for 5 minutes at 2000 RPM following which 250µL of supernatant was retrieved from each vial and stored in a freezer (-80°*C*)**.** The tubes were then replenished with 250 µl of PBS and placed back into the incubator shaker at -37°*C*. After 28 days of daily supernatant collection, a micro bicinchoninic acid (µBCA) assay was conducted to measure the daily OPG protein release (Thermoscientific) as per manufacturer's instructions. Based on release from initial prototypes, we made adjustments to the OPG formulations in the type and molecular weight of the PEG used to achieve the desired rate of 1mg/kg of OPG over 28 days (as determined by the

current effective dose of 1 mg/kg OPG in 350 gm rats). For 1mg/kg to be delivered over 28 days, the target release rate of an average male rate is 11.7µg/day.

Thin Film Device Fabrication

Given that thin film devices have never been used in an oral environment, a specific design was required that would remain securely in place and be positioned away from biting forces, while successfully delivering the drug into the permeable tissues. The film needed to be large enough to be loaded with sufficient amounts of the drug. Thus, to maximize the loading of OPG and surface area of the film, the device was designed (Fig. 10) to spread across the palatal surface due to this being the largest non-biting surface in the rat oral cavity. To fabricate the films, slot casting methods were used. First, a glass plate was solvent cleaned with acetone, methanol, and isopropyl alcohol (IPA). Second, A 1:1 ratio of PEG and PCL were dissolved in the amount of 150 mg per ml of2,2,2,-trifluoroethanol (TFE). Lastly, the PEG/PCL/TFE solution was poured onto the cleaned glass plate and casting was performed with a clearance applicator with pre-determined thicknesses. With the clearance applicator, a 15 mil film was made. The casted thin film was then placed in milli-q water for 24 hours to dissolve the PEG solution (i.e., porogen leaching) to form pores within the thin film. The porogen leached films were subjected to laser cutting to obtain the specific design for the rat oral cavity with a diameter of 11.3 mm wide including the arms, while the drug releasing portion was designed to be 4.4 mm to fit the roof of the mouth. A total of 95 ug of OPG at a concentration of 18.85 mg/mL was loaded into the films. The film was designed to have unidirectional drug transport with its porous side facing the palate to

allow drug release into the palatal mucosa, while the nonporous side composed of only PCL to face the oral cavity. For placement, it was bonded to the buccal surfaces of the second and third molars using dental adhesives. After spin-casting and laser cutting, the films were loaded with OPG and heat sealed for immersion into PBS. The daily supernatant was collected to measure the release of OPG using μBCA as described previously for the PEG delivery system.

Results

In our first trial, we determined the OPG loading concentrations in PEG that would result in appropriate release of desired concentrations of OPG over a 7 day period. The microspheres were constructed with three concentrations of OPG at 1/10, 1/50 and 1/100 dilution of the original OPG of 18.85 mg/ml. The protein release from the microspheres was determined every 24 hours using micro-BCA for 7 days (Fig. 11A). On the first day, an average of 176.00 ug/mL of OPG was released from the 1/10 diluted OPG solution, 101.11 ug/mL for the 1/50 solution, and 114.18 ug/mL for the 1/100 solution. By day 4, the release began showing a more steady trend with 53.3ug/mL, 18.63 ug/mL, and 19.05 ug/mL of OPG being released for the 1/10, 1/50, and 1/100 solutions, respectively. Over the duration of 7 days on the average a total of 522.41 ug, 239.60, and 281.70 ug of OPG were released respectively for the highest to lowest concentrations of OPG utilized (Fig. 11B). The graph shows a release burst followed by a decreased release of protein over 7 days.

After identifying that the highest concentration of OPG used (1/10 dilution or 188 ug/ml) resulted in release in appropriate range of OPG, but given that this occurred relatively quickly over 7 days, we made modifications to the PEG in an attempt to achieve a slower and more sustained OPG release. For this experiment OPG (1/10 dilutions) was encapsulated in microspheres fabricated with 50%PEGDMA750 and 50% PEGDMA200 (1.5%) and the release kinetics determined over 7 days (Fig. 12). This modification resulted in a more constant release in a range of 18-28 ug/mL with standard deviations ranging from 3.3 to 8.23 revealing a diminished burst release seen when using PEG by itself.

Thin Film Release Assay

The thin films were first tested with adhesives to determine the effects of the bond on the device. The devices were weighed on a milligram scale at 1, 3, 7, 14 and 38 days after adhesive bonding. The average weight of the film remained stable at 15mg before and after each allotted time period. No significant reduction in weight signifying degradation of the films was observed (Fig. 13).

Scanning electron microscopy was used to examine the surface topography to confirm the integrity of the thin films after adhesive binding (Fig. 14A). The adhesives did not seep into the opposing side of the film (Fig. 14B), and a clear junction was noted on the designated adhesive region of the thin film and remained clear of the remainder of the film (Fig. 14C).

Thin Film Protein Release Assay #1

The devices were loaded with 80-110 ug of OPG. No wash step was performed. The limitations of OPG loaded were due to size limitations of the device. On day 1, the three devices released 78ug, 88ug, and 99ug of OPG respectively (Fig. 15). In the following 3 days, less than 5ug was released per day.

Thin film protein release assay #2

In the second thin film release assay, some modifications were made. The neck size of the film device was increased (Fig. 16) to allow for improved loading and prevention of load loss as seen in the results from the first round of release assays. In

addition, a pre-wash step was included for rinsing the films before elution. Three devices were loaded with 80-105 ug of OPG (Fig. 17). Device 1 released 40ug of OPG on day 1 and then tapered off below 10 ug for the following 3 days. Device 2 experienced a steady increase in protein release until day 2, having released a total of 60 ug over three days. However on day 3, a negligible amount of protein was released, followed by another peak release of 25 ug on day 4. Device 3 experienced most of the release on day 1 of the experiment, followed by 3 days of negligible protein release.

Given the inconsistent results of protein release experienced with the thin films, scanning electron microscopy was performed to examine the binding surfaces of the films (Fig. 18). Porous structures were identified between the two surfaces of the thin film indicating the sealing process is impaired by human error.

Discussion

In these studies, we fabricated and tested *in vitro* potential drug delivery methods for use in orthodontic therapy including mitigating post-orthodontic relapse, which is a multifactorial and remains poorly understood. While we can attempt to predict which of our patients is more likely to experience relapse and take measures towards prevention, much of this process currently remains out of our control given that we currently rely on patient compliance during the several critical months it takes for bone stabilization to occur. Although the turnover and maturation of bone is only one of the components of retention, it is an important one and as seen in the medical field, we have developed successful means to promote it. Aside from orthodontic purposes, there is value in producing new methods to promote bone turnover and maturation in other aspects of dentistry, such as periodontology. In periodontology, methods for inhibiting the degeneration of bone and promoting regeneration of bone is of high value given the nature of the problems seen in patients with periodontal disease.

While we have developed methods to deliver OPG locally and found success in its ability to promote bone stabilization, we further aimed to explore methods of sustained release as well as noninvasive methods of delivery in this study. Specifically, we used the validated dose of OPG required to significantly improve post-orthodontic stability to develop methods for delivering that dose in a controlled manner over a long duration of time. We used microspheres composed of PEG which are known to be more effective than PLGA in controlling the delivery of proteins due to their chemical composition and interaction with the body. The specific composition of PEG needed for our purposes was unknown and required a few trials before it could be determined. In

our first attempt we had to test two variables— the concentration of OPG that would produce appropriate release profiles with PEG to achieve our target release per day of 0.5 mg/kg, and the type of PEG that would produce a consistent controlled release. We used three concentrations of OPG (1/10, 1/50, 1/100) and a high molecular weight (750) PEG. The results seen in Figure 10 reveal a burst release experienced by all three groups. Each concentration group experienced between 51.11-126 ug more protein released than the target release of 50 ug on day 1. This burst release was likely due to the inability of the microspheres to contain the OPG. In addition, because all three concentrations of OPG showed similar release kinetics, and therefore, appropriate reactions with PEG, we were able to eliminate the need of multiple concentrations of OPG for the subsequent trials.

The retention of OPG in the microspheres required improvement in order to achieve the target release of 50ug/day. Previous studies have shown that reducing the molecular weight of PEG can reduce the diffusivity of proteins from the microspheres.[40] Decreasing molecular weight of PEG allows us to increase the number of PEG molecules, which in return, increases crosslinkable double bonds. The increased strength from the double bonds provides a stronger structure which reduces diffusivity.[40] Thus, lower molecular weight PEG microspheres expel their contents in a more controlled fashion. For this reason, we combined 50% molecular weight PEG200(1.5%) with 50% of the original higher molecular weight PEG (750) to create a mixture for the second batch of hydrogels. The data resulting from this modification revealed a much more steady release of OPG ranging between 18-28ug/day. Although the amount released per day was lower than our target release of 50 ug/kg, we find this to be

beneficial because although much of the bone maturation processes occur within the first 30 days, the process continues for up to 6 months. With this in mind, it is likely beneficial to have the lower dose spread over a longer period of time. Thus this delivery method holds promise for future studies to confirm their release kinetics in vitro and efficacy tests in vivo.

Thin Films

To begin, we needed to ensure that the dental adhesives which would be used with the thin film design would not dissolve the films due to their acidic nature. To do so, the adhesives were placed on the films and co-incubated for 1-30 days in PBS. They were removed from PBS and lyophilized on days 1,3,7,14, and 30 to remove the residual PBS and to weigh the films. Because the results revealed no changes in weight, we can conclude that there was no degradation of the thin film devices. This confirmation allowed us to move forward with the current design of the thin film device.

To visualize and confirm the integrity of the thin films adhesive binding, scanning electron microscopy was used. Figure 14A shows an SEM that was taken of the side of the films where the dental adhesives were placed. Here, we can appreciate the architecture of the dental adhesives for a frame of reference for when we examine the opposite side of the film (Figure 14B) without dental adhesives. This image allows us to confirm that the adhesives do not seep through the film and that the coincubation of the films with the adhesives is not affected by the adhesives. Lastly, In Figure 14C, a clear junction was noted on the designated adhesive region of the thin film and remained

clear of the remainder of the film. This again confirms that the adhesive stays in place, and does not seep through to the rest of the film.

The maximum amount of OPG we were able to load into the thin film devices was 80-110ug of OPG. This presented as our first limitation to thin film protein delivery. Although we designed the largest possible thin film for a rat's intraoral cavity, we need to be able to load a significant amount more in order to reach our target release of 50 ug/kg/day.

In our first release assay, no wash step was performed. On day 1, the three devices released 78ug, 88ug, and 99ug of OPG respectively. During the following 3 days, less than 5ug was released per day. These results indicate that much of the protein was lost on the surface of the thin films indicated by the burst release. To improve the release and limit the loss of OPG, a wash step was added. In addition, the loading of the OPG into the films was a difficult step due to the physical constraints of the neck size of the films.

In the second protein release assay, the neck size of the films were increased and the films were prewashed before elution. Three devices were loaded with 90ug of protein and assayed for protein release for 4 days. On day 0, the amount of protein lost in the wash was observed to be between 5ug-25ug. Device 1 released most of its protein (60ug) on day 1, followed by insignificant amounts of protein for the remaining days. Device 2 presented the most promising release of protein with 20-30ug/day, except for day 3 when a very small amount of protein was released (<5ug). Device 3 presented very similarly to device 1 by minimal protein being lost in the wash step (<5ug) and the majority of protein released on day 1 (60ug). In this assay, we largely

improved the loss of the protein by increasing the neck size and including the wash step. However, the varying results of the assay suggest that the technique of thin film fabrication requires improvement. To determine which step required improvement, we examined the seal of the thin films by SEM. In Figure 18, the interface where the porous and nonporous sides of the films are sealed together is shown. This image reveals pores in that interface suggesting flaws in the sealing step of thin film fabrication. This step is currently a limitation in nanotechnology and needs further optimization.

Conclusion

While we have developed methods to deliver OPG locally and found success in its ability to promote bone stabilization, we further explored methods of sustained release as well as noninvasive methods of delivery in this study. Osteoprotegerin released by 50%PEGDMA250(1.5%) 50%PEGDMA750 microspheres could provide a more controlled release than the previously studied methods of release. Thin film devices may also provide a completely non-invasive approach to delivering OPG, but require further improvements in the sealing process.

Future Directions

The OPG release in 50%PEGDMA250(1.5%) 50%PEGDMA750 microspheres should be confirmed with a 30 day release assay followed by an ELISA to confirm the protein did not denature during microsphere fabrication. Following this confirmation, the microspheres should be tested in the animal model we have proposed in Figures 1, 2 and 7. The thin films remain a viable option but require extensive improvements in the sealing process. In addition, the amount of protein loaded into the microspheres needs to be increased in order to meet the target release of protein per day. It is possible that flash freezing the proteins to reconstitute them at a higher concentration before loading the films may be one solution.

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List of Figures

Figure 1. Appliance placement. Niti coil spring from maxillary molars to incisors.

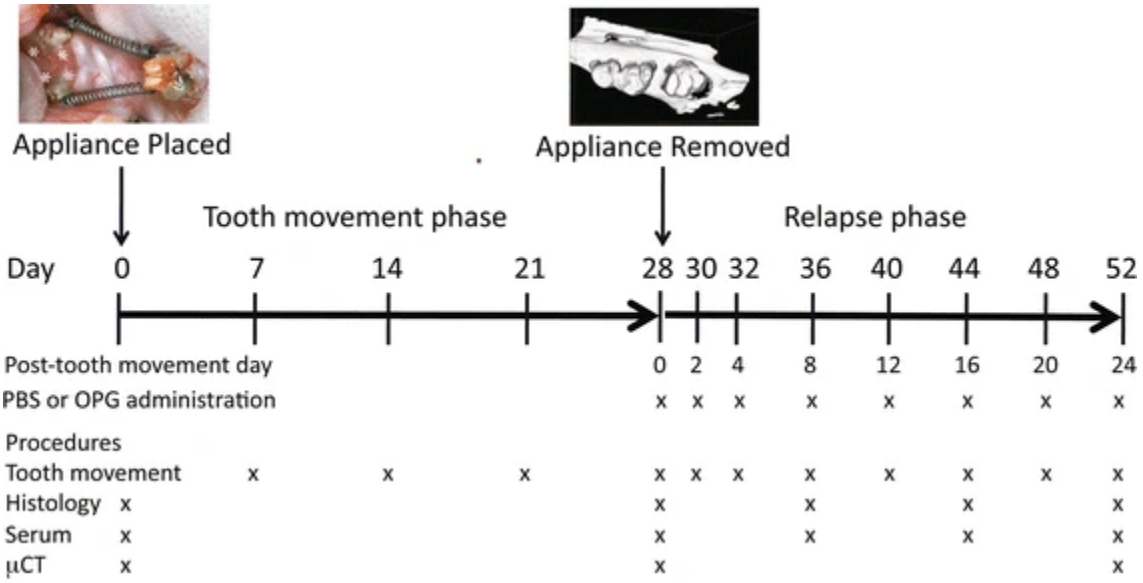


Figure 2: Animal study timeline of procedures

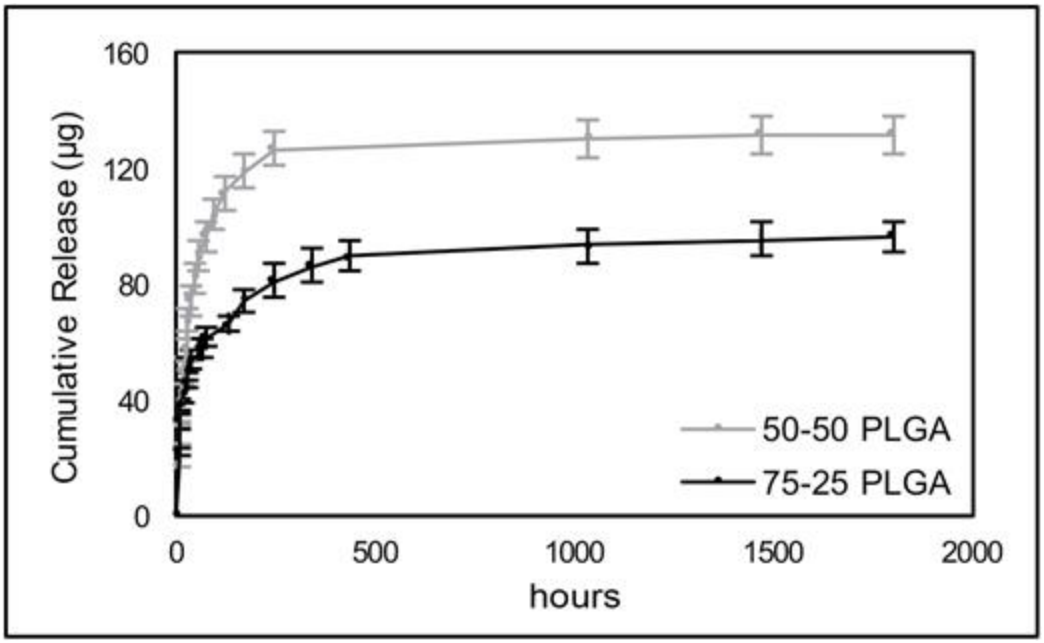


Figure 3. PLGA microsphere cumulative release assay

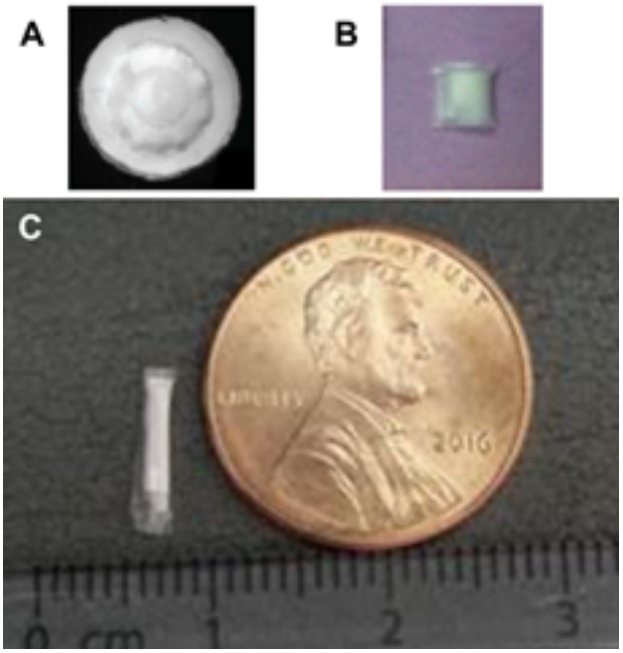


Figure 4. Examples of thin film device types and sizes.

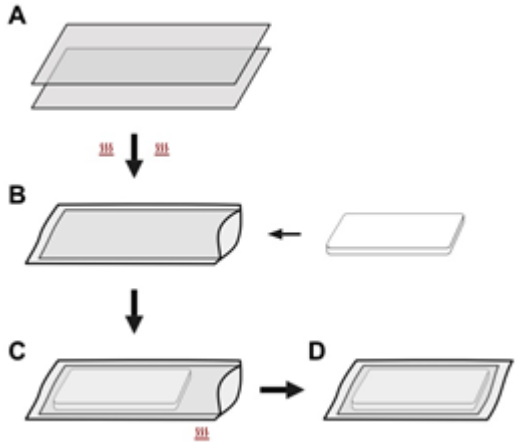


Figure 5. Fabrication of planar thin film devices

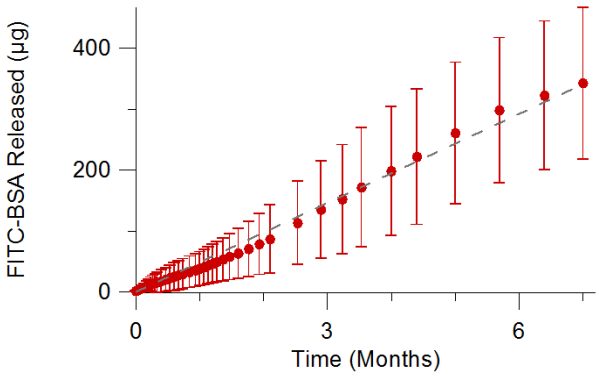


Figure 6. In vitro release of fluoroscein isothiocyanate-labeled bovine serum albumin

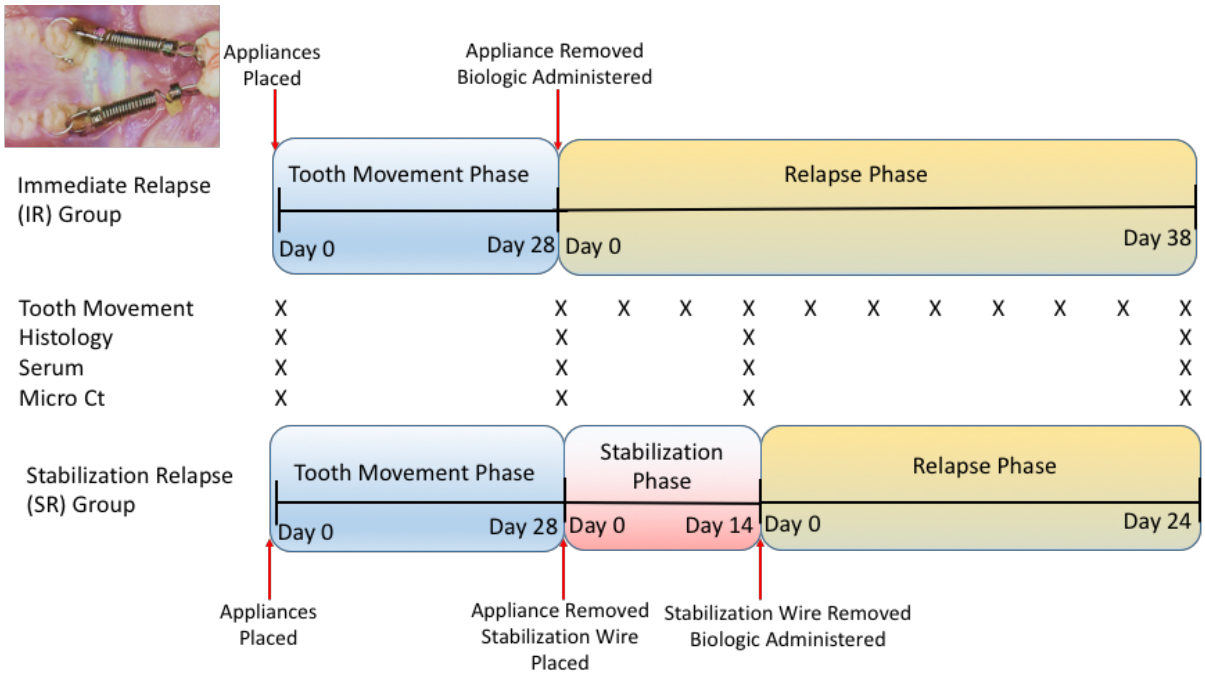


Figure 7. Schematic of study design for in vivo testing of the efficacy of osteogenic or anti-osteolytic drug delivery systems.

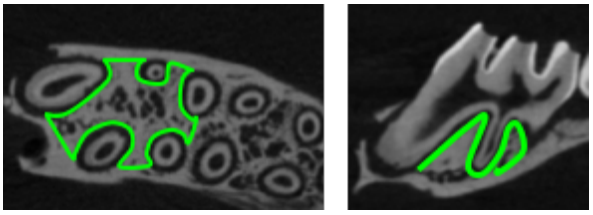


Figure 8. Representation of 3D volume to be utilized for determining intraradicular bone quality using micro-CT.

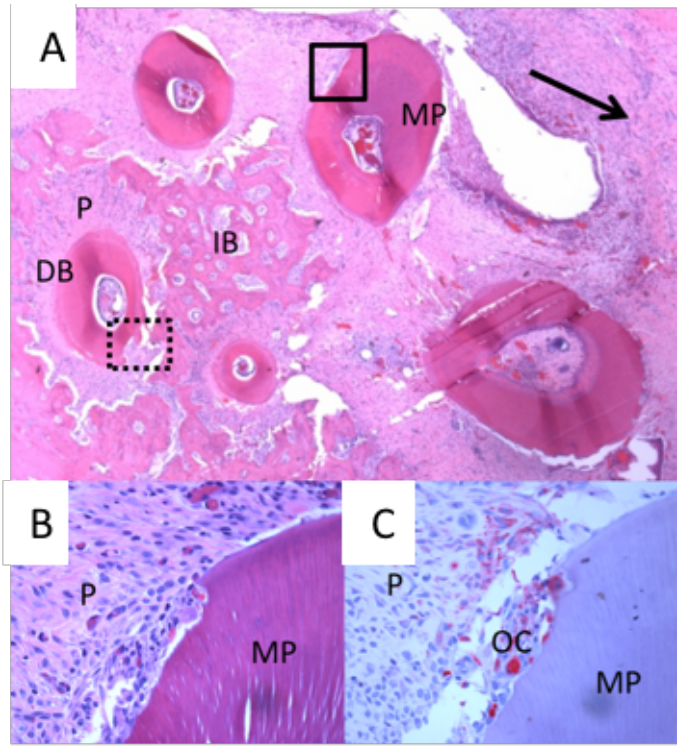


Figure 9: Axial section through the rat first molar roots and maxillary alveolus. (A) Low power HE staining to demonstrating five roots and intraradicular bone sites that will be analyzed for bone formation, and bone and root resorption. (B) High power image showing root resorption pit. (C) TRAP positive staining of multinucleated cells will be quantitated for bone and root resorptive activity. (IB=intraradicular bone; P=periodontal ligament; MP=mesiopalatal root; DB=distobuccal root; OC=TRAP positive osteoclasts)

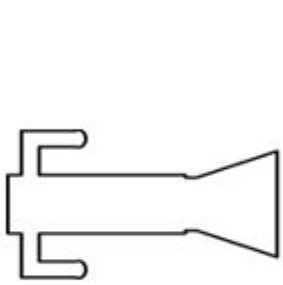


Figure 10. Thin film device for placement on rat palate.

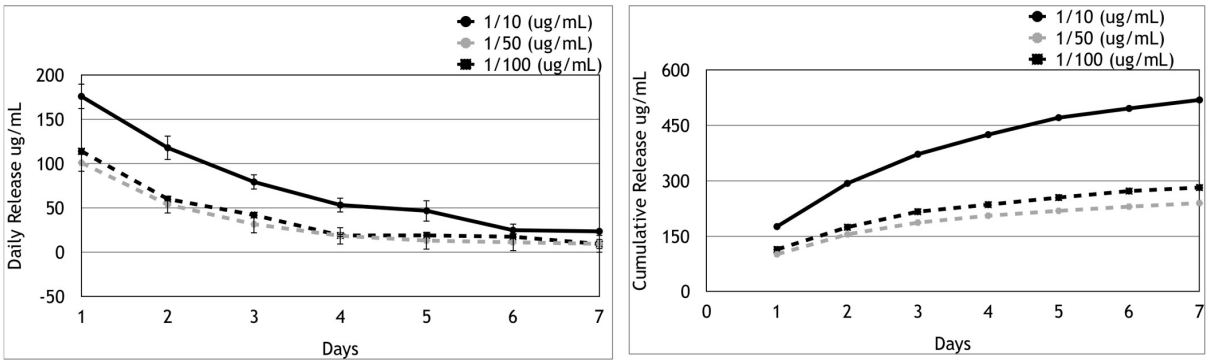


Figure 11. Daily (A) and cumulative (B) release of protein (OPG) from PEG microspheres for three concentrations of OPG (1/10 dilution or 1885 ug/ml; 1/50 dilution or 377 ug/ml and 1/100 dilution of 188.5 ug/ml OPG) over a 7 day duration.

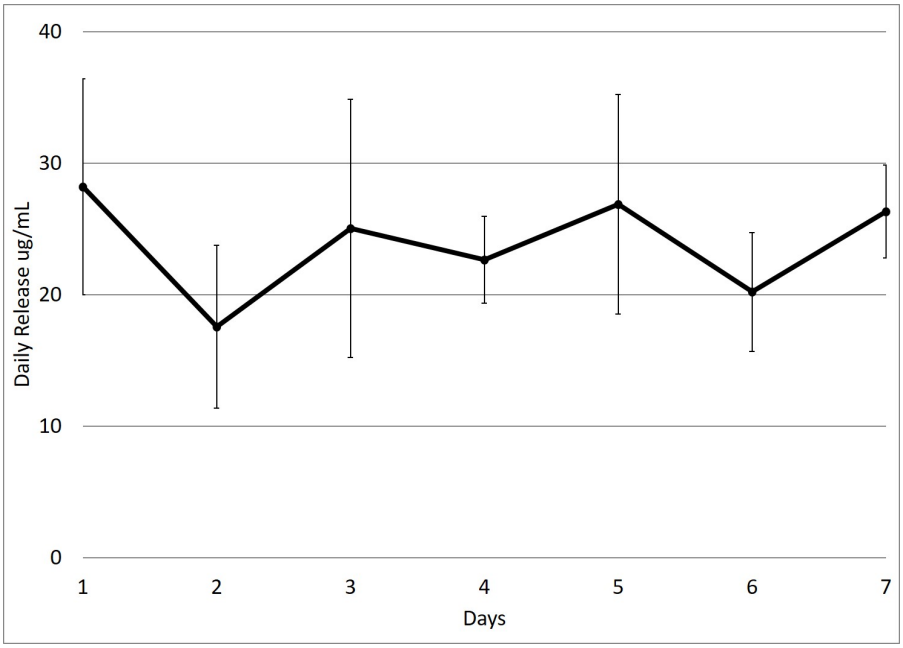


Figure 12. Daily release of OPG from 250%PEGDMA750 and 50% PEGDMA200(1.5%) microspheres over 7 days using 1/10 dilution of OPG.

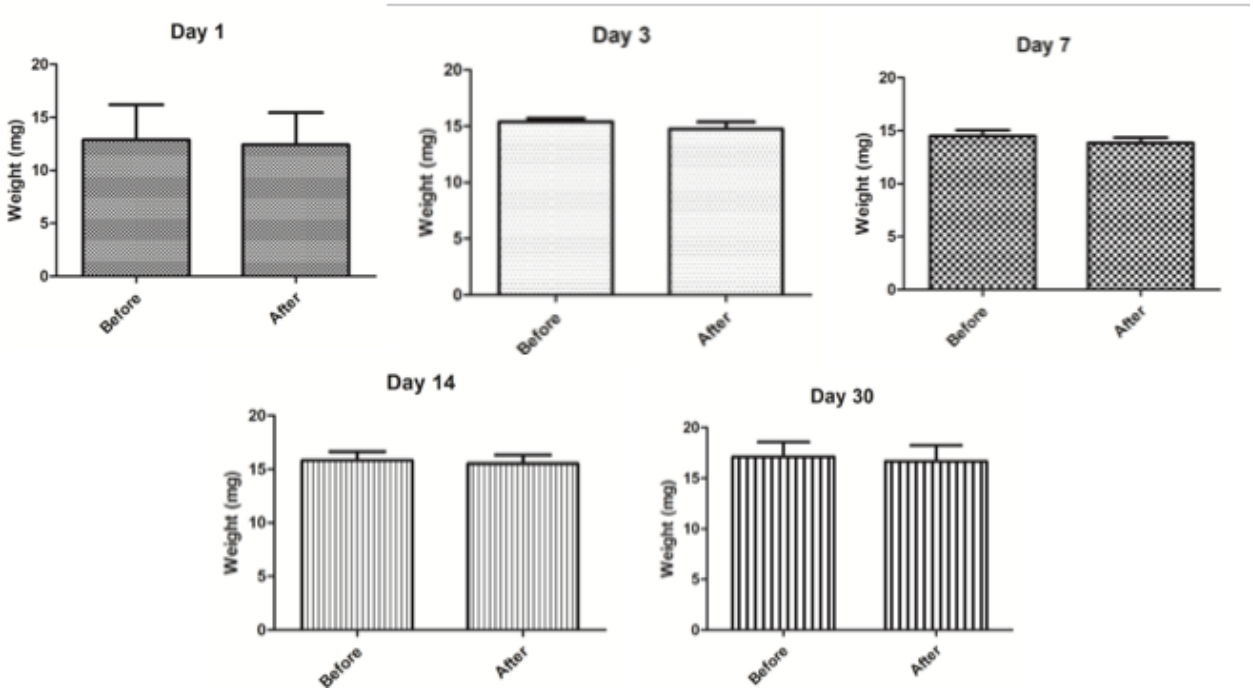
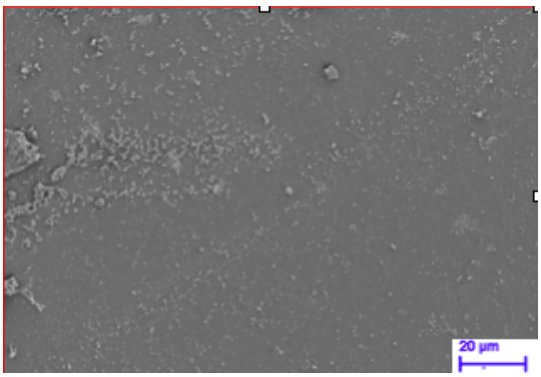
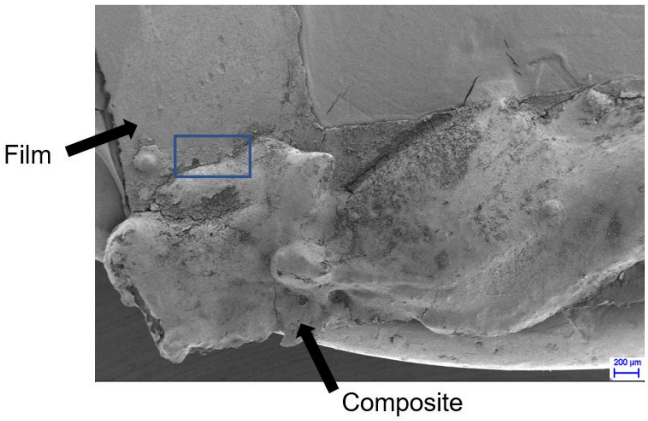


Figure 13. Coincubation of Thin Films with adhesives does not affect device weight.

14.1



14.2



14.3

Figure 14: SEM photomicrographs of composite-Thin Film interface. 14.1. Composite architecture on composite side of Thin Film device. 14.2 Architecture of thin film on non-composite side of Thin Film device. 14.3. High power photomicrograph of composite and thin film interface.

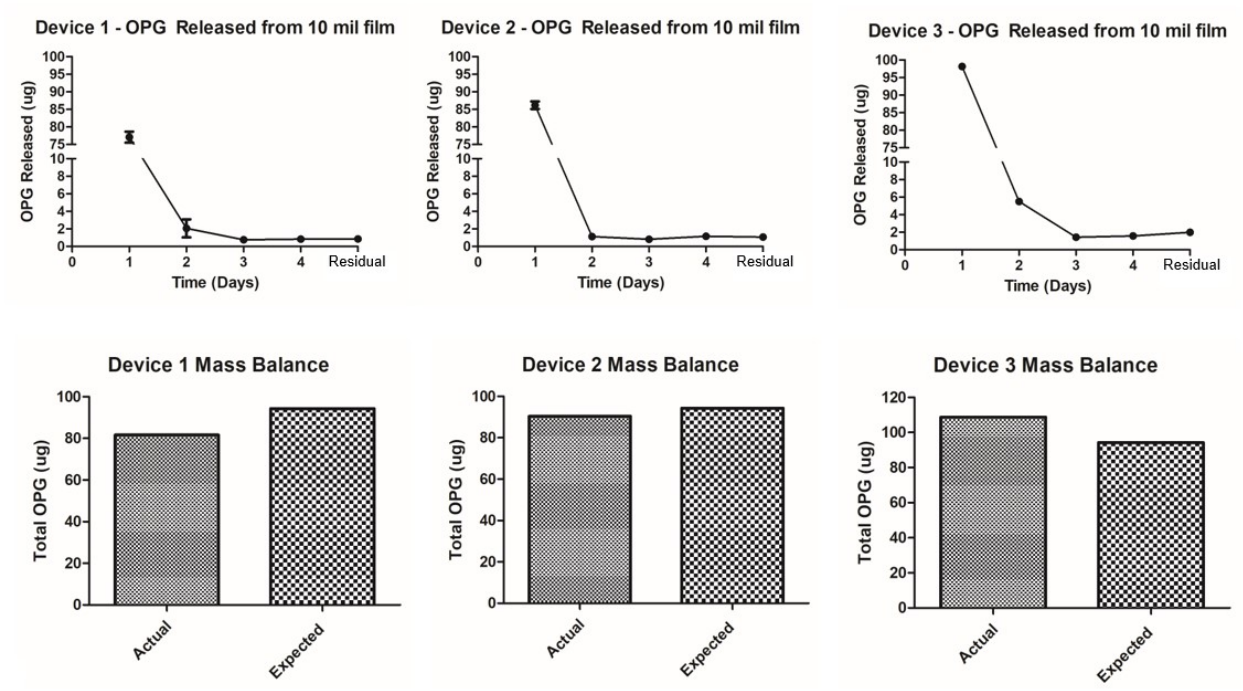


Figure 15. Thin Film Release Assay 1

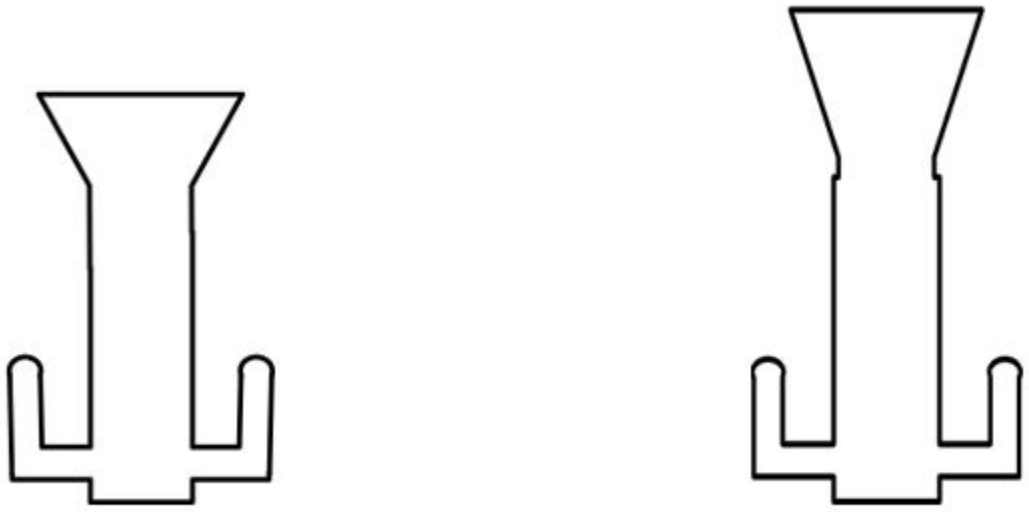


Figure 16. Neck size increased in 2nd thin film device design.

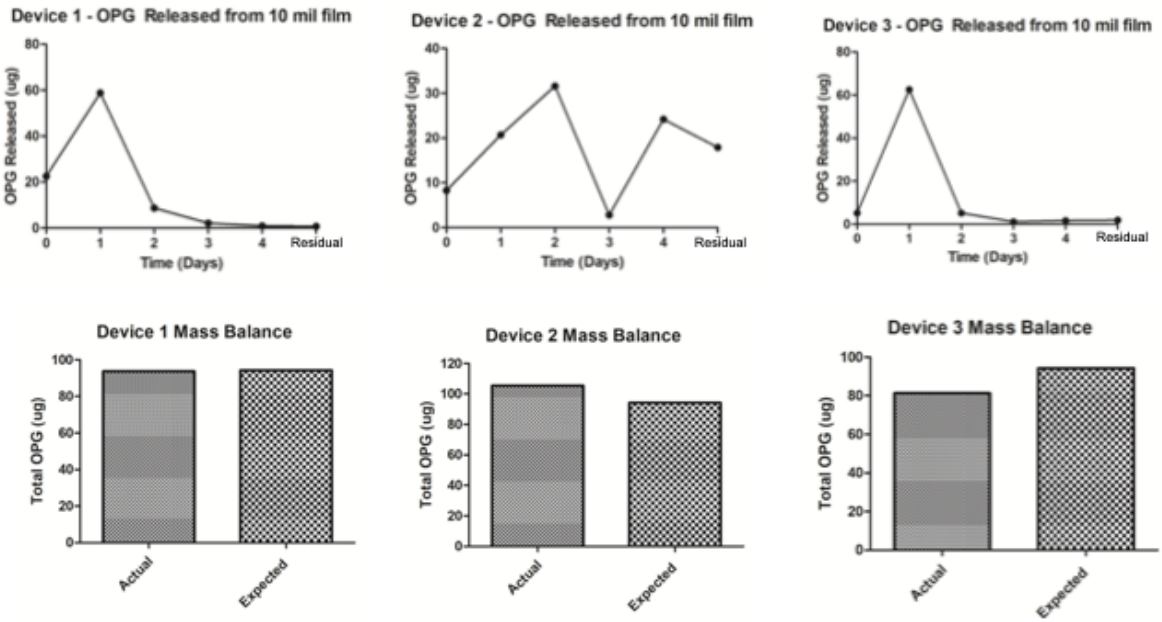


Figure 17. Thin film release assay 2.

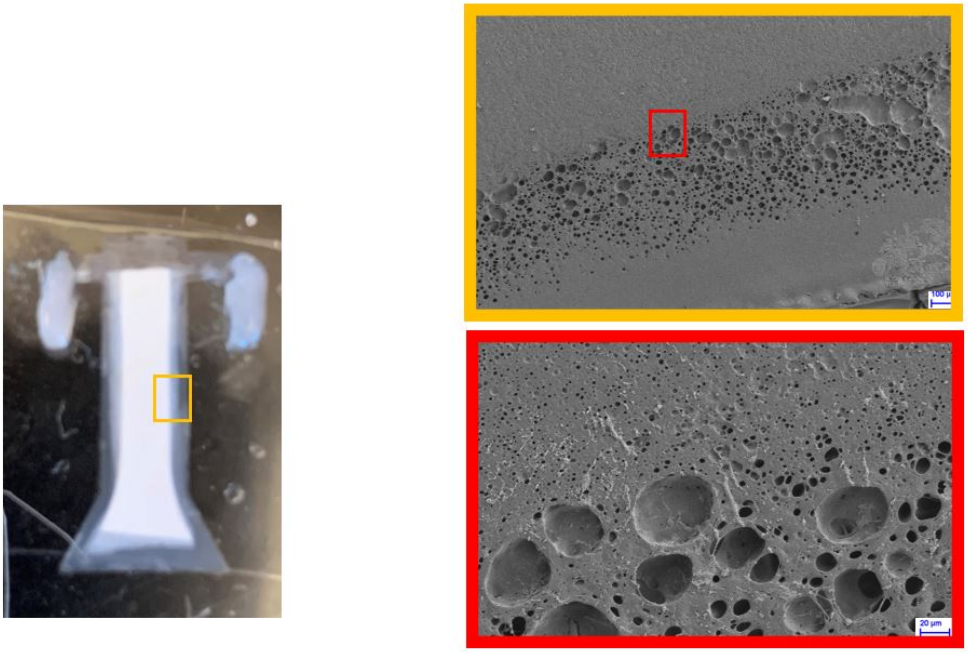


Figure 18. Porous and non-porous interface of fabricated Thin Films demonstrate inadequate sealing of the device.

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